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Effect of Dibutylnitrosamine and Saccharin on Glutamyl Transpeptidase-Positive Foci and Liver Cancer

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An attempt was made to evaluate whether the simultaneous administration of the urinary bladder tumor promoter, saccharin, and the substance being tested for carcinogenicity could be developed into a rapid and efficient bioassay for bladder carcinogens. Dibutylnitrosamine (DBN) a bladder and—to a lesser extent—liver carcinogen, was used as the test substance. The attempted evaluation failed because of the high incidence of liver cancer in the rats that simultaneously received DBN and saccharin. The simultaneous administration of 5% sodium saccharin in the diet and 0.02% DBN in the drinking water of rats for 26 weeks resulted in an 81% DBN in the drinking water of male rats for 26 weeks resulted in an 81% incidence of both hyperplastic nodules and hepatocellular carcinomas. A much lower incidence of 17 and 3% for hyperplastic nodules and hepatocellular carcinomas, respectively, was present in the rats that received only DBN, and no tumors were present in the liver of rats that received only saccharin. The liver of the rats that received both DBN and saccharin compared to those that received only DBN also had an increased incidence of γ-glutamyltranspeptidase (GGTase)-positive foci at 4, 8 and 26 weeks of treatment. The presence of GGTase activity in hyperplastic nodules and hepatocellular carcinomas and the association of the increased incidence of GGTase-positive foci with the increased incidence of tumors are consistent with a precursor relationship of foci to hyperplastic nodules and hepatocellular carcinomas. Saccharin did not increase the size of GGTase-positive foci, indicating that saccharin is not an hepatic tumor promotor. The increased incidence of DBN initiated GGTase-positive foci and tumors that resulted from the simultaneous administration for saccharin, implicates an hepatic cocarcinogenic activity for saccharin.

Areas of GGTase-positive hepatocytes were induced in zone 1 by DBN, saccharin and DBN and saccharin at 4 and 8 but not 26 weeks. These zonal areas of induced GGTase activity are different from GGTase-positive foci. The induction of zonal GGTase activity by saccharin indicated that saccharin at the high dose of 5% in the diet can cause hepatotoxicity and/or bile ductule proliferation. It is proposed that a regenerative response to the hepatotoxicity could result in an increased efficacy of DBN initiation by increasing the fixation of DBN adduct in DNA.

Introduction

γ-Glutamyltranspeptidase (GGTase)-positive foci are proposed to be preneoplastic lesions in rat livers (*1-3*). The incidence of GGTase-positive foci has been proposed to be associated with the extent of the initiation of the neoplastic progression and with the ultimate incidence of hyperplastic nodules and hepatocellular carcinomas (*1-3*). The screening of chemicals for tumor promoting and cocarcinogenic activity by demonstration of their ability to enhance the incidence of GGTase-positive foci is predicated on the capability of the incidence of foci to predict the cancer incidence. A tumor promoter would act after initiation to stimulate the growth of the foci so that there is an earlier increase in foci and tumor incidence. A cocarcinogen would act during initiation to increase the efficacy of initiation as indicated by an increased number of GGTase-positive foci.

Saccharin 1, 2-benzisothiazol-3(2H)-one-1,1-dioxide, (CAS No. 81-07-2) is a weak urinary bladder carcinogen in rats (*1-4*). Chemical carcinogenesis in the urinary bladder has been divided into the two stages of initiation and promotion. Sodium saccharin ad-

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ministered in the diet has been shown to promote the incidence of urinary bladder cancer in rats that were previously initiated with a subcarcinogenic dose of either methylnitrosourea administered intravesically (*4-6*), FANFT, *N*-4-(5-nitro-2-furyl)-2-thiazolylformamide administered in their diet (*7*) or *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) in their drinking water (*8*). In mouse skin (*9,10*) and urinary bladder (*8*), tumor promoters have been shown also to possess cocarcinogenic activity. The simultaneous administration of the carcinogen and the promoter resulted in an enhanced and more rapid appearance of tumors than occurred in the two stage bioassay consisting of the carcinogen followed by the promoter. The study reported in this communication describes an attempt to develop a rapid and efficient bioassay for bladder carcinogens that consists of administering the substance being tested for carcinogenicity simultaneously with the bladder tumor promoter, saccharin. This communication also describes the effect of saccharin concurrently administered with dibutylnitrosamine (DBN) on the relationship of DBN induced GGTase-positive foci to hyperplastic nodules and hepatocellular carcinomas.

Materials and Methods

Chemicals

Sodium saccharin synthesized by the Maumee Procedure was purchased from Sigma Chemical Company (St. Louis, MO) and added at a concentration of 5% by weight to AIN-76 semipurified diet purchased from ICN Nutritional Biochemicals (Cleveland, OH). The sodium saccharin as expected for Maumee synthesized saccharin, was determined by gas chromatography to contain undetectable levels of either *o*-toluenesulfonamide or *p*-toluenesulfonamide. Dibutylnitrosamine was purchased from Eastman Kodak Company (Rochester, NY) and *N*-γ-L-glutamyl-4-methoxy-2-naphthylamide from Bachem (Torrence, CA).

Animals

Male Fischer 344 rats were purchased from Charles River Laboratories, Inc. (Portage, MI) and were 7 to 8 weeks of age at the start of the experiment. The animals were maintained in accordance with the standards set forth in the literature (*11*). They received their drinking water and food *ad libitum*.

Experimental Design

The experimental design consisted of four treatment groups. Group A received the control AIN-76 diet; Group B, AIN-76 diet and 0.02% DBN in their drinking water; Group C, 5% sodium saccharin in the AIN-76 diet; and Group D, 5% sodium saccharin in the AIN-76 diet and 0.02% in their drinking water. Rats were sacrificed from each of the four groups at 4, 8, and 26 weeks of treatment. The experiment was terminated at 26 weeks because of the high mortality rate in Group D.

Histopathology

At the termination of the experiment, the rats were killed by decapitation and necropsied. The urinary bladder was inflated with 10% buffered formalin and the inflated bladder and liver fixed in 10% buffered formalin. After the tissues were embedded in paraffin, sections obtained from each of four different lobes of the liver and a longitudinal section from each of the two halves of the urinary bladder were examined.

For evaluation of GGTase activity, tissues blocks approximately 10 × 10 × 2 mm were taken from three different lobes, rapidly frozen in optimum cutting temperature (OCT) compound on dry ice and stored at -80°C for up to 6 months. Cryostat sections (8 μm) were mounted on slides, air-dried, and stained for γ-glutamyltranspeptidase activity according to the method described by Rutenburg et al. (*12*). Nuclei were counterstained with hematoxylin. Only GGTase-positive foci of nine cells (nuclei) or greater were scored. Figure 1 depicts a

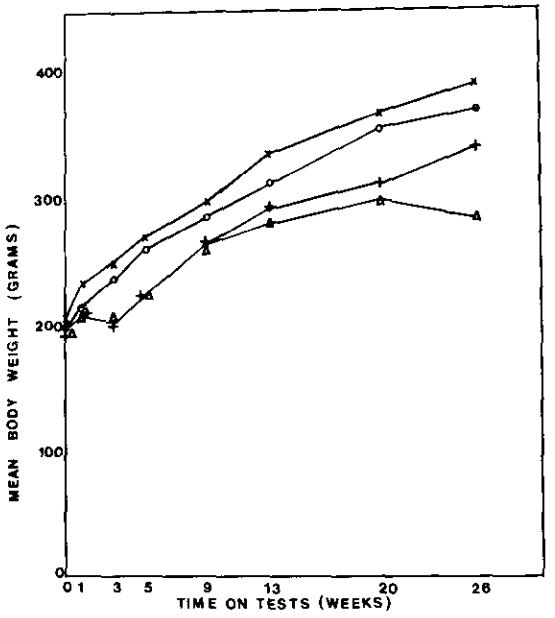

FIGURE 2. Average body weights of rats in the four treatment groups from week 0 to 26 of the experiment: (×) Group A, controls; (O) Group B, DBN; (+) Group C, saccharin; (▲) Group D, DBN and saccharin.

typical GGTase-positive focus. The liver sections, GGTase-positive foci and areas of GGTase-positive hepatocytes were sized by projection of the image onto the surface of a digitizer.

Results

Toxicity

The effect of treatment on the body weights of the rats is presented in Figure 2. The rate of gain in body weight over the course of the experiment was not affected by DBN, but sodium saccharin caused a decreased rate of gain. The body weight of the rats that simultaneously received saccharin and DBN were the lowest of all the groups and appeared to decrease after 20 weeks, during which time a large number of rats died. This high mortality rate in Group D resulted in the termination of the experiment at week 26.

The amount of drinking water consumed by the rats of each group was determined on a biweekly basis. Over the 26-week course of the experiment the mean percentage ± standard error in drinking water consumption determined as mL/100 mg body weight relative to control (Group 4) was for Group B 97 ± 10, group C, 135 ± 10, and Group D, 134 ± 12. The consumption of water by Group D was therefore on the average 37% greater than Group B.

The liver weights and their percentage of the body weight are presented in Table 1. DBN did not alter the percentage of the body weight represented by the liver, while sodium saccharin decreased the contribution of the liver to the body weight. Upon histological evaluation, the livers from the control and DBN treated rats contained hepatocytes with fat vacuoles that were diffusely dispersed, mainly in zone 1 of Rappaport. The livers from saccharin-treated rats (Group C) lacked these fat vacuoles which might explain their lower percent of the body weight. The livers of the rats from Group D represented an apparent, but not statistically significant, higher percentage of the body weight. This apparent increase in the relative weight of the liver probably resulted from the presence of the numerous large tumors. The rats in Group D also had a complete lack of body fat.

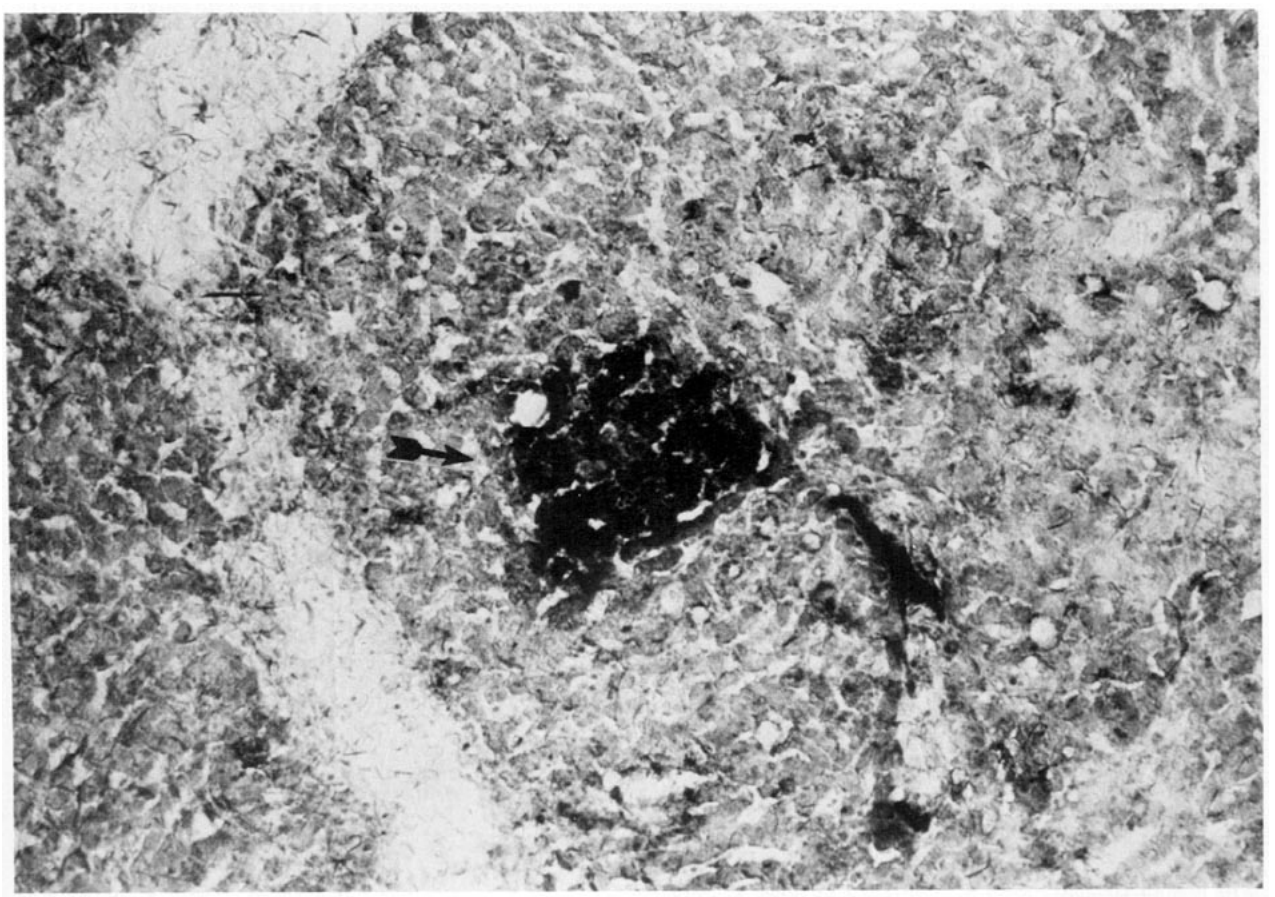

FIGURE 1. GGTase-positive focus. The liver section was stained for γ-glutamyltranspeptidase activity and counterstained with hematoxylin. × 100.

Initiation of GGTase-Positive Foci

The incidence of GGTase-positive foci was higher in Group D when compared to Group A, B or C (Table 2). At 26 weeks, Group B also had an increased incidence of foci compared to Groups A or C; however, throughout the course of the study the incidence of foci in Group D was much greater than Group B. At no time during the experiment did sodium saccharin administered by itself (Group C) significantly increase the incidence of foci. Therefore, DBN induced the formation of GGTase- positive foci and the coadministration of saccharin greatly enhanced the response to DBN.

The size distribution of the GGTase-positive foci was determined at 26 weeks for Group B and D (Table 3). The size distribution of the foci was divided into logarithmic intervals from 0.0-0.03 to >3 mm^2. The foci in Groups B and D had the same size distribution. Even the percentage of foci greater than 3 mm^2, which probably represents hyperplastic nodules, was the same in Groups B and D. Therefore, the concurrent administration of saccharin with DBN did not increase the rate of growth of GGTase-positive foci, so that saccharin did not appear to promote DBN hepatocarcinogenesis.

Tumorigenicity

Tumors were found only in the liver of rats from Groups B and D (Fig. 3 and Table 2). The coadministration of sodium saccharin in the diet greatly increased the incidence of hyperplastic nodules and hepatocellular carcinomas that were induced by DBN. The hyperplastic nodules and hepatocellular carcinomas were positive for GGTase activity. The carcinomas were of the trabecular type. In Group B, animals which received only DBN, there were 16

Table 1. Body and liver weights at 26 weeks for the various experimental groups.

Group	Treatment	N^a	Body weight, g[b]	Liver weight, g[b]	Liver weight, % of body weight
A	Control	11	391 ± 8.8	12.6 ± 0.85	3.21 ± 0.17
B	DBN	29	367 ± 8.3	11.4 ± 0.41	3.10 ± 0.07
C	Saccharin	36	337 ± 4.2	9.47 ± 0.21	2.81 ± 0.04*
D	Saccharin	21	282 ± 10.7	10.7 ± 0.73	3.78 ± 0.20**

[a] *N represents the number of animals at the termination of the study.*
[b] Values are means ± SE.
* *Different from Group A by the Student t test with p = 0.0017.*
** Different from Group A by the Student *t* test with $p = 0.085$.

Table 2. Induction by DBN and saccharin of GGTase-positive foci and tumors in rat liver.

Group	Treatment	GGTase-positive foci/cm^2 4 weeks[a]	8 weeks[a]	26 weeks[a]	Hyperplastic nodules Animals[b]	%	Carcinoma Animals[b]	%
A	Control	0.43 ± 0.30(14)	1.63 ± 0.81(10)	0.31 ± 0.31(10)	0	0	0	0
B	DBN	1.88 ± 0.45(12)	2.19 ± 0.72(8)	5.03 ± 1.06(29)*	5	17	1	3
C	Saccharin	1.95 ± 0.61(13)	1.57 ± 0.54(9)	0.23 ± 0.23(36)	0	0	0	0
D	DBN + saccharin	6.26 ± 1.69(11)*,+	9.19 ± 1.84(9)*,+	31.31 ± 4.65(21)*,+	17	81	17	81

[a] Foci results are mean ± standard error for the number of animals in parentheses.
[b] Results are the number of animals at 26 weeks with tumors among the animals observed for foci at 26 weeks.
* Results are different from Group A by the Student *t* test with $p<0.01$.
\+ Results are different from Group B by the Student *t* test with $p<0.01$.

Table 3. Size distribution of GGTase-positive foci at 26 weeks.

Group	Treatment	N^a	Size distribution of GGT-ase positive foci, % 0.0-0.03 mm^2	0.03-0.1 mm^2	0.1-0.3 mm^2	0.3-1 mm^2	1-3 mm^2	3 mm^2
B.	DBN	44	14[c]	41	30	9	5	2
D.	DBN + Saccharin	244	14	43	29	8	2	3

[a] *N* equals the number of foci evaluated.

hyperplastic nodules among five of 29 (17%) rats and two carcinomas in one of 29 (3%) rats, and in Group D animals which received both DBN and saccharin, there were 106 hyperplastic nodules among 17 of 21 (81%) rats and 81 carcinomas among 17 of 21 (81%) rats. Therefore, DBN and not saccharin possessed the ability to initiate tumors and the coadministration of saccharin with DBN greatly enhanced the tumorigenic potency of DBN.

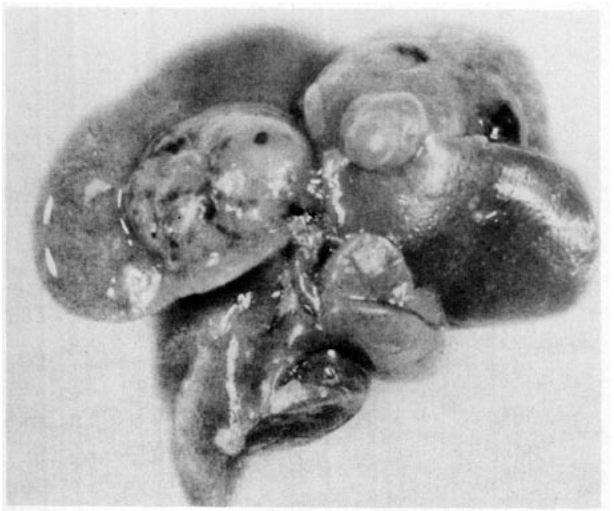

FIGURE 3. Liver from a rat simultaneously treated for 26 weeks with *N*-dibutylnitrosamine and saccharin (Group D) that exhibited hyperplastic nodules and hepatocellular carcinomas.

Urinary bladder tumors, either benign or malignant, were not observed in any of the rats of the four groups. There was simple focal/and/nodular-papillary hyperplasia of the bladder transitional epithelium in the animals that received either DBN (Group B) or saccharin and DBN (Group D; Table 4). The hyperplastic response was associated with the administration of DBN and not the administration of the sodium saccharin. The coadministration of sodium saccharin increased both the incidence and severity of the hyperplastic response to DBN in the urinary bladder.

Areas (Zone 1) of GGTase-Positive Hepatocytes

During the course of this study, we observed an induction in Groups B, C and D of GGTase activity in hepatocytes in Zone 1 of the liver as defined by Rappaport (Fig. 4). These zonal areas of GGTase-positive hepatocytes were distinct from foci in that they appeared to radiate out from most, if not all, triads of a section, while foci were dispersed randomly in all three zones. The percentage of the liver containing GGTase activity in zone 1 hepatocytes is presented in Table 5. At 4 and 8 weeks, an increase in GGTase activity was observed in Groups B, C and D. The order of efficacy was DBN + saccharin > saccharin > DBN. The coadministration of DBN and saccharin appeared to be synergistic, that is, greater than the addition of the potencies of DBN

Table 4. Lesions of the urinary bladder.

Group	Treatment	*N*[a]	Number of animals showing hyperplasia[b]		
			Simple Focal	Nodular and Papillary	Total
A.	Control	10	0	0	0
B.	DBN	20	3(15)	4(20)	7(35)
C.	Saccharin	25	1(4)	0	0
D.	Saccharin + DBN	10	3(30)	4(40)	7(70)

[a] N represents the number of bladders examined by light microscopy. The remaining bladders were used for histochemical and electron microscopic examination.

[b] Results are the number of animals examined that exhibited the lesions. Animals that exhibited both nodular and single focal hyperplasia were scored for the more advanced nodular lesion. The numbers in parentheses are the percentage of the animals that exhibited the lesion.

Table 5. Induction by DBN and saccharin of GGTase-positive hepatocytes.

Group	Treatment	GGTase-positive hepatocytes, % of liver[a]		
		4 weeks	8 weeks	26 weeks
A.	Control	0.66 ± 0.24(14)[a]	0.19 ± 0.10(10)	0 ± 0(10)
B.	DBN	2.6 ± 0.53(12)	2.9 ± 0.73(9)	0.05 ± 0.03(29)
C.	Saccharin	7.4 ± 1.4(13)	5.1 ± 1.2(9)	0 ± 0(36)
D.	Saccharin + DBN	13.7 ± 1.4(11	16.3 ± 1.8(9)	0.92 ± 0.43(21)

[a] Results are means ± standard error for the number of animals in parentheses.

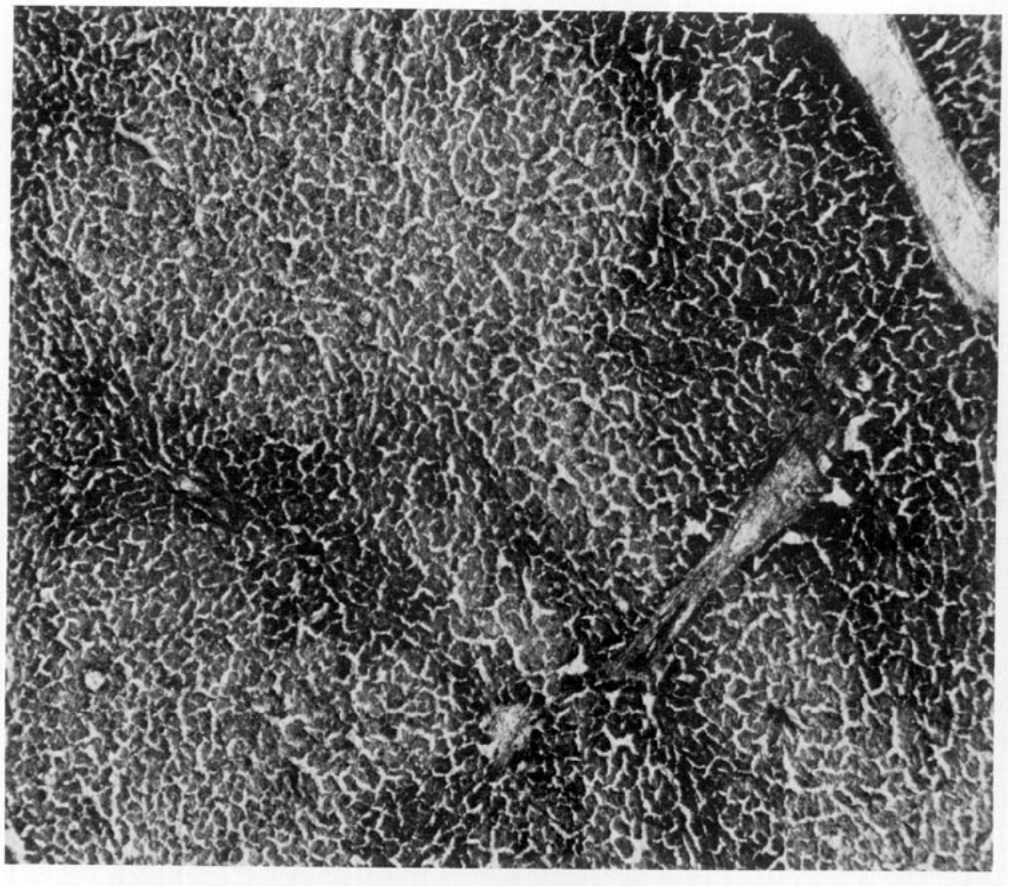

FIGURE 4. Zonal areas of induced GGTase activity. The liver section was stained for GGTase activity and counterstained with hematoxylin. The zonal areas of induced GGTase activity appear to radiate out from the triads. × 40.

and saccharin. Even in the presence of continuous treatment with DBN and saccharin, by 26 weeks, the zonal areas of GGTase activity had regressed. GGTase-positive foci on the other hand, continued to increase in number and size.

Discussion

The first step in the neoplastic progression of chemical carcinogenesis is the initiation of the target cell. Initiation is believed to start with the covalent binding of the carcinogen or one of its metabolites to DNA. Replication of the DNA then completes the initiation step by fixation of the alteration in the genetic code of the daughter strand. DNA repair prior to fixation of the alteration prevents initiation. Cellular replication of the genetically altered and initiated cell results in a focus of cells expressing an altered phenotype. The acquisition of GGTase activity is one of the altered phenotypes used to identify these foci of transformed hepatocytes (*1-3*). The incidence of GGTase-positive foci would, therefore, be an indication of the extent to which a carcinogen has initiated carcinogenesis.

Dibutylnitrosamine induced GGTase-positive foci, hyperplastic nodules and hepatocellular carcinomas. The nodules and carcinomas also contained GTase activity. When saccharin was administered concurrently with DBN, there was an increased incidence of foci, nodules and carcinomas. This is consistent with a precursor relationship of GGTase-positive foci to hyperplastic nodules and hepatocellular carcinomas. Therefore, it appears that the induced incidence of GGTase-positive foci by a chemical would predict the hepatocarcinogenicity of the chemical.

Tumor promoters act after initiation has been completed to stimulate the neoplastic progression so that tumors appear earlier. The appearance of GGTase-positive foci occurs at the end of the initiation step so that the effect of promoters would be to enhance the growth and progression of foci to cancer. The concurrent administration of saccharin with DBN did not increase the size of GGTase-positive foci indicating that saccharin does not promote

hepatocarcinogenesis. Ito et al. (*13*) have demonstrated that saccharin is not a liver tumor promoter in *N*-(4-hydroxybutyl) nitrosamine-initiated rats.

One explanation for the enhancement of the incidence of DBN induced GGTase-positive foci, hyperplastic nodules and hepatocellular carcinomas is that the rats administered saccharin drank more water and therefore received a larger dose of DBN. Nakanishi et al. (*8*), in their cocarcinogenicity study of saccharin with BBN, observed a similar 50% increase in drinking water consumption in rats that received saccharin with or without BBN in their drinking water. It is unlikely, however, that the 37% increase in the dose of DBN as the result of concurrent administration of saccharin would have caused such a large (2600%) increase in hepatocellular carcinomas. Also, in rats, DBN is a much more potent bladder carcinogen than liver carcinogen (*14-20*). Therefore, if saccharin was acting solely to increase the dosage of DBN, one would expect to observe an increase in bladder cancer. We did not detect any bladder tumors in the rats that received concurrently DBN and saccharin. It would appear that the effect of concurrently administered saccharin to increase the carcinogenicity of DBN was much greater in the liver than the bladder and therefore was not simply the result of an increase in dose. Other explanations of why saccharin was cocarcinogenic toward DBN could be that saccharin stimulated the hepatic uptake and metabolism of DBN or induced an increase in cellular proliferation prior to the repair of the DBN adducts in DNA.

In support of the theory that a saccharin-induced increase in cellular proliferation and fixation of DBN adducts in DNA is the fact that saccharin induced GGTase activity in the hepatocytes of zone 1. These zonal areas of induced GGTase activity were distinct from foci in their appearance, location in the lobule, and regression by 26 weeks, even in the presence of the inducer. Zonal induction of GGTase activity is probably the source of the serum GGTase activity used to indicate liver toxicity so that, the high dose of 5% sodium saccharin in the diet would appear to exert zone 1 toxicity in rat liver. Zonal necrosis was observed in the liver in response to saccharin by Chowaniec and Hicks (*21*). A regenerative response to this necrosis could increase the rate of cellular replication resulting in an increased fixation of the DBN alteration in DNA. This proposed increased fixation of the DBN alteration in the DNA would result in an increased efficacy of DBN initiation of GGTase-positive foci and tumors.

The premature termination of the study due to the high mortality in the rats that received both DBN and saccharin occurred before the appearance of DBN-induced urinary bladder tumors. DBN did induce simple focal, nodular and papillary hyperplasia and the concurrent administration of saccharin increased both the incidence and severity of the lesions. The concurrent administration of sodium saccharin in the diet with *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (BBN) in the drinking water enhanced the hyperplastic and tumor response to BBN (*8*). In our investigation and Nakanishi's (*8*) study, the 37% and 50% increase in drinking water consumption resulting from saccharin administration obscured the interpretation that saccharin is a cocarcinogen. Therefore, further evaluation of the possible cocarcinogenic activity of saccharin is necessary.

Summary

Consistent with the proposed precursor relationship of GGTase-positive foci to hepatocarcinogenesis, the induction of foci by DBN was associated with the induction of hyperplastic nodules and hepatocellular carcinoma and the concurrent administration of sodium saccharin in the diet with DBN in the drinking water increased the tumorigenic response in the liver to DBN.

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